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SCREENING OF BIO-ACTIVE COMPOUNDS AND ANTICANCER ACTIVITY OF *Punica granatum* L.

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ABSTRACT

In the present study to evaluate the anticancer activity and determination of phytocommpounds in *Punica granatum* peel using GCMS. The phytochemical screening of Punica granatum peel showed that the presence of flavonoids, phenolics, tannin, anthroquinones, saponins, protein, glycosides, phlobatannins, alkaloids, and while steroids and triterpenoids were absent. GC-MS study of *P. granatum* peel indicates presence of 25 compounds a. The prevailing compounds *Punica granatum* peels were 2-propanone, 1-[(3-methyl-2-buten), 9-Octadecenoic Acid (Z)-, Hexadecanoic Acid, Cyclododecasiloxane, Tetracosam,Methyl Stearate ,Cyclononasiloxane, Octadecamethyl, Hexasiloxane and Tetradecamethyl. P. granatum peel concentration increases there is an increase in the cell growth inhibition but is found to be highest inhibition with only 91.11 % growth inhibition at 500µg. The IC_{50} value was 155.40 µg/ml. The anticancer activities of active plants are probably due to presence of phytochemicals as Flavonoids. Over all the *P. granatum* peel extract as a source of phytochemicals and possessed potential anticancer activity in cancer cell line.

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INTRODUCTION

Cancer is a growing public problem whose estimated worldwide new incidence is about 6 million cases per year. It is the second major cause of deaths after cardiovascular diseases. Cancer is a general term applied of series of malignant diseases that may affect different parts of the body. These diseases are characterized by a rapid and uncontrolled formation of abnormal cells, which may mass together to form a growth or tumor or proliferate throughout the body, initiating abnormal growth at other sites. If the process is not arrested, it may progress until it causes the death of the organism. These cells are born due to imbalance in the body and by correcting this imbalance the cancer may be treated (Siegel and Zhu, 2009).

Breast cancer is the most common female cancer worldwide representing nearly a quarter (23%) of all cancers in women (GBD, 2015). The global burden of breast cancer is expected to cross 2 million by the year 2030, with growing proportions from developing countries. Although age-standardised incidence rates in India are lower than in the United Kingdom (UK) (25.8 versus 95 per 100,000), mortality rates are nearly as high (12.7 versus 17.1 per 100,000, respectively) as those of the UK. Breast cancer incidence rates within India display a 3–4-fold variation across the country, with the highest rates observed in the Northeast and in

major metropolitan cities such as Mumbai and New Delhi. Reasons for this variation include differences in demographic (e.g., education), reproductive (e.g., age at first child and number of children), anthropometric (e.g., adiposity) and life style factors (e.g., tobacco smoking and alcohol use) (Gupta *et al*., 2015).

Medicinal plants possess antioxidant properties, leading to anticancer activities (Pandey *et al.*, 2006). Plants contain several phytochemicals, which possess strong antioxidant activities. The antioxidants may prevent and cure cancer and other diseases by protecting the cells from damage caused by 'free radicals' the highly reactive oxygen compounds. Thus consuming a diet rich in antioxidant plant foods (e.g., fruits and vegetables) will provide a milieu of phytochemicals, nonnutritive substances in plants that possess healthprotective effects. Many naturally occurring substances present in the human diet have been identified as potential chemopreventive agents and consuming relatively large amounts of vegetables and fruits can prevent the development of cancer. Compared with meat eaters, most, but not all, studies have found that vegetarians are less likely to be diagnosed with cancer. Vegetarians have also been shown to have stronger immune function, possibly explaining why they may be partially protected against cancer. Many plant-derived products have been reported to exhibit potent antitumour activity against several rodent and human cancer cell lines (Lin *et al.*, 1996). The medicinal value of the chosen plant *Punica granatum* peel has been extensively worked out. However, its therapeutic efficacy in *in vitro* anticancer activity has not been evaluated. In the present study to evaluate the anticancer activity and determination of phytocommpounds in *Punica granatum* peel using GCMS.

MATERIALS AND METHODS

Chemicals

All the chemicals used were of analytical grade and were obtained from Glaxo Laboratories, Mumbai, India and Sisco Research Laboratories, Mumbai, India.

Plant material and Preparation of extract

The peels of the *Punica granatum* were collected from the Thirumanu, Ariyalur district, Tamil Nadu, during the month of May 2016. The *Punica granatum* peels were cut into small pieces and shade dried at room temperature for 15 days. The powdered plants were used for the preparation of extract. The dried materials was ground into make a fine powder and used for extraction. Three hundred grams (300g) of the powered plants were extracted with methanol (70%) for 24 hours. A semi solid extract was obtained after complete elimination of alcohol under reduced pressure. The percentage of yiels was 16. The extract was stored in a refrigerator until used. The extract contains both polar and non-polar phytocomponents.

Preliminary phytochemicals screening Chemical tests were carried out on the alcoholic extract using standard procedures to identify the preliminary phytochemical screening following the methodology of Sofowara (1993), Trease and Evans (1989) and Harborne (1973).

GC MS Analysis

GC MS analysis was carried out on Shimadzu 2010 plus comprising a AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer instrument employing the following conditions: column RTX 5Ms (Column diameter is 0..32mm, column length is 30m, column thickness 0.50µm), operating in electron impact mode at 70eV; Helium gas (99.999%) was used as carrier gas at a constant flow of 1.73 ml /min and an injection volume of 0.5 µI was employed (split ratio of 10:1) injector temperature 270 ºC; ion-source temperature 200 ºC. The oven temperature was programmed from 40ºC (isothermal for 2 min), with an increase of 8 ºC/min, to 150ºC, then 8ºC/min to 250ºC, ending with a 20min isothermal at 280ºC. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 40 to 450 Da. Total GC running time is 51.25min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a TurboMass Ver 5.2.0 (Srinivasan *et al*., 2013).

Identification of components

Interpretation on GCMS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained (Dukes, 2013).

ANTICANCER AND CYTOTOXIC ASSAY

Preparation of extracts

Different concentrations of (10, 20, 30, 40 and 50µg/ml) peel extract was prepared. These concentrations were used for anticancer activity.

Anticancer assay

Antiproliferative and cytotoxic assay was evaluated by the MTT reduction assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] (Mosmann, 1983; Monks *et al*., 1991). The monolayer cells were detached and single cell suspensions were made using trypsin-ethylenediaminetetraacetic acid (EDTA). A hemocytometer was used to count the viable cells and the cell suspension was diluted with a medium containing 5% FBS in order to obtain final density of 1x105 cells/ml. 96-well plates at plating density of 10,000 cells/well were seeded with one hundred microlitres per well of cell suspension and incubated for cell attachment at 37° C, 5% CO_2, 95% air and 100% relative humidity. Aliquots of 100 µl of different concentrations of leaf and bark extracts (10, 20, 30, 40 and 50µg/ml) dissolved in DMSO (1%) were added to the appropriate wells already containing 100 µl of medium, resulted the required final sample concentrations for 48h at 37°C, 5% CO_2, 95% air and 100% relative humidity. After 48h of incubation, to each well 20µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) phosphate- buffered saline solution was added and incubated at 37°C for 4 h. Then, 100µl of 0.1% DMSO is added to each well to dissolve the MTT metabolic product. Then the plate is shaken at 150 rpm for 5 min. Viable cells were determined by the absorbance at 570nm. Measurements were performed and the concentration required for inhibition Concentration (IC_{50}) was determined graphically. The absorbance at 570nm was measured with a UV- Spectrophotometer. The medium without samples served as control and triplicate was maintained for all concentrations. The effect of the samples on the proliferation of MCF-7 was expressed as the % cell viability & % Cell growth inhibition using the following formulas:

% Cell viability =

Abs 570 of treated cells / Abs 570 of control cells × 100%.

% Cell growth inhibition =

[100 - Abs (sample) /Abs (control)] x100.

Statistical Analysis:

Values were expressed as mean ± SD for six rats in the each group and statistical significant differences between mean values were determined by one way analysis of variance (ANOVA) followed by the Tukey's test for multiple comparisons. The results were statistically analyzed by Graphpad Instat Software (Graphpad Software, San Diego, CA, USA) version 3 was used and $p< 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Medicinal plants are assuming greater importance in the primary health care of individuals and communities in many developing countries. Medicinal plants are believed to be much safer and proved earlier in the treatment of various ailments. Medicinal plants are a major source of biodynamic compounds of therapeutic values (Ashis, 2003). In the present study was carried out on the plant sample revealed the presence of medicinally active constituents. The phytochemical characters of the *Punica granatum* investigated and summarized in Table-1. The phytochemical screening *Punica granatum* showed that the presence of flavonoids, phenolics, tannin, anthroquinones, saponins, protein, glycosides, phlobatannins, alkaloids, and while steroids and triterpenoids were absent.

Table 1: Phytochemical screening of *Punica granatum*

S.NO	SECONDARY METABOLIES	RESULTS
1	Tannin	+
2	Phlobatannis	+
3	Saponnins	+
4	Flavonoids	+
5	Sterods	-
6	Terpenoids	+
7	Triterpenoids	-
8	Alkaloids	+
9	Carbohydrate	++
10	Protein	+
11	Anthroquinone	+
12	Polyphenol	++
13	Glycoside	+

(+) = Presence; (-) Absence; (++) = Moderate concentration

Sangeetha and Jayaprakash (2015) investigated the phytochemical screening of *Punica granatum* Linn. Peel extracts. The peel powder of *P. granatum* was extracted with respective solvents namely aqueous, ethanol, acetone, petroleum ether and chloroform. Qualitative phytochemical screening of *P. granatum* peel extracts were assessed by standard methods. All the phytochemical constituents tested were present in aqueous extract of *P. granatum* peel except glycosides and anthocyanin. It was noted that ethanolic peel extract of *P. granatum* showed the presence of all phytochemical constituents except tannins, glycosides and anthocyanin. The chloroform peel extract showed only presence of phytochemical constituents out of. Petroleum ether extract of *P. granatum* peel showed the presence of saponins and phenols alone. All the phytochemical constituents tested were present in acetone extract of *P. granatum* peel except alkaloids, saponins and anthocyanin.

GC-MS study of *P. granatum* peel indicates presence of 25 compounds a (Fig -1, Table -2). The prevailing compounds *Punica granatum* peels were 2-propanone, 1-[(3-methyl-2-buten), 3,3-dimethoxy-2-butanone, 1,3-Dioxolane-4-methanol, 2-ethyl-, Sulfurous acid, hexyl octyl este,1-Octyl trifluoroacetate , Decane, 2,3,4-trimethyl- ,Cyclohexasiloxane, Dodecamethyl- ,Cycloheptasiloxane, Tetradecamethyl- ,9-Octadecenoic Acid (Z)-,1,3-Diphenyl-1,3,5,5-Tetramethyl-C,1,3-Diphenyl-1,3,5,5-Tetramethyl-C,Heptasiloxane, 2,6,10-Trimethyl,14-Ethylene-14-PE,Phosphine Oxide, Bis(Pentamethylphenyl)- ,Hexadecanoic Acid, Methyl Ester , [Dodecanoyl(Methyl) Amino]Aceti, Dibutyl Phthalate $$ 1,2-Benzenedicarboxylic A, 9-Octadecenoic Acid (Z)-, Methyl E, Cyclododecasiloxane, Tetracosam,Methyl Stearate ,Cyclononasiloxane, Octadecamethyl- ,2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16,18,18,2 Dodecamethylcyclohexasiloxane, Hexasiloxane and Tetradecamethyl. The biological activity and other structure of the identified compounds were represented in (Table -3).

Fig 1: Chromatogram obtained from the GC/MS with the extract of *Punica granatum* peels

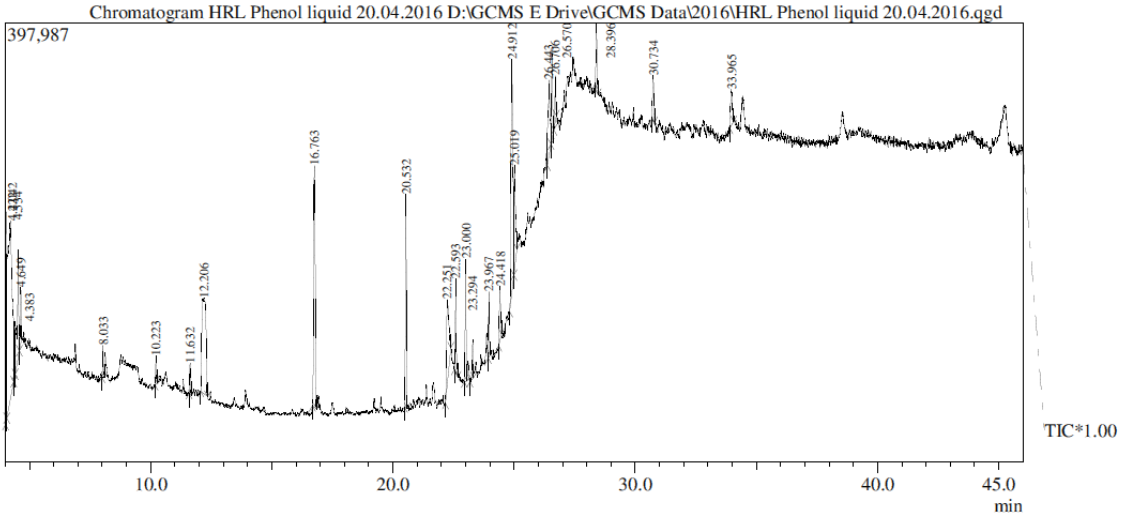


Table 2: Phyto- Components identified in ethanolic extract of *Punica granatum* peels (GC- MS Study)

Peak	R.Time	Area %	Height %	Name
1	4.042	4.49	8.76	Acetic acid, butyl ester $$ n-Butyl acetate
2	4.222	4.067	21.09	N-Methylglycine $$ Sarcosine $$ CH3NHCH
3	4.383	1.70	2.35	2-Propanone, 1-[(3-Methyl-2-Buten
4	4.534	4.28	4.53	3,3-Dimethoxy-2-butanone

5	4.649	1.27	2.37	1,3-Dioxolane-4-methanol, 2-ethyl-
6	8.033	0.67	1.33	Sulfurous acid, hexyl octyl este
7	10.223	0.59	1.18	1-Octyl trifluoroacetate $$ Octyl trifluoroaceta
8	11.632	0.70	1.32	Decane, 2,3,4-trimethyl- $$ 2,3,4-Trimethyld
9	12.206	9.40	4.01	Cyclohexasiloxane, dodecamethyl-
10	16.763	9.57	10.10	Cycloheptasiloxane, tetradecamethyl- $$
11	20.532	4.68	8.94	Cyclooctasiloxane, hexadecamethyl-
12	22.251	5.02	3.44	9-Octadecenoic Acid (Z)- $$
13	22.593	2.23	3.89	1,3-Diphenyl-1,3,5,5-Tetramethyl-C
14	23.000	2.59	5.11	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13
15	23.294	0.98	1.62	2,6,10-TRIMETHYL,14-Ethylene-14-PE
16	23.967	1.27	2.71	Phosphine oxide, bis(pentamethylphenyl)-
17	24.418	1.51	2.22	Hexadecanoic acid, methyl ester $$ Palmitic a
18	24.912	12.26	9.74	[Dodecanoyl(Methyl)Amino]Aceti
19	25.019	3.98	4.34	Dibutyl phthalate $$ 1,2-Benzenedicarboxylic a
20	26.443	3.77	3.15	9-Octadecenoic ACID (Z)-, Methyl E
21	26.570	2.10	3.51	Cyclododecasiloxane, Tetracosam
22	26.706	1.03	1.86	Methyl stearate $$ Octadecanoic acid, methyl
23	28.396	1.84	2.73	Cyclononasiloxane, octadecamethyl- $$
24	30.734	1.32	1.88	2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16,18,18,2
25	33.965	1.67	1.50	Hexasiloxane, Tetradecamethyl-
		100.00	100.00	

Table 3: Biological activity of phyto-components in *Punica granatum* peels

NAME	BIOLOGICAL ACTIVITY**
Acetic acid, butyl ester	No activity
N-methylglycine	Anti microbial
2-propanone, 1-[(3-methyl-2-buten)	Anti inflamatory
3,3-dimethoxy-2-butanone	Antioxident,Anti cancer
1,3-Dioxolane-4-methanol, 2-ethyl-	Anti inflamotary,Antibaterial
Sulfurous acid, hexyl octyl este	Antipyretic,Antisalmonella,Antiseptic,Antistaphylococcus
1-Octyl trifluoroacetate	Antioxident,Antiinflammatory,Anticancer
Decane, 2,3,4-trimethyl-	**Anti-Tumor**,Analgesic Anti Bacterial,Sedative
Cyclohexasiloxane, Dodecamethyl-	Preservative
Cycloheptasiloxane, Tetradecamethyl-	Antioxidant,Flavor,Hypocholesterolemic
9-Octadecenoic Acid (Z)-	Antimicrobial,Antifouling
1,3-Diphenyl-1,3,5,5-Tetramethyl-C	Fungicide,Anti-Inflammatory,**Antitumor**
1,3-Diphenyl-1,3,5,5-Tetramethyl-C	Antimicrobial,Antiinflammatory
Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13	Used Inflavouring Of Spirit Drinks

2,6,10-Trimethyl,14-Ethylene-14-PE	No Activity Reported
Phosphine Oxide, Bis(Pentamethylphenyl)-	Antimicrobial
Hexadecanoic Acid, Methyl Ester	Antioxidant,Nematiside,Pesticide,Anti-Androgenic,5-Alpha Redeustase,Hemolytic
[Dodecanoyl(Methyl)Amino]Aceti	Used As Softnes,Used In Preparation Perfumes & Cosmatics,Raincoats,Boots
Dibutyl Phthalate $$ 1,2-Benzenedicarboxylic A	Used In Textiles Asdyestuffs,Glass Making
9-Octadecenoic Acid (Z)-, Methyl E	Antihypertensive, Increase HDL Decrease
Cyclododecasiloxane, Tetracosam	Anticancer,Antimicrobial,Antioxident
Methyl Stearate	**Antitumor**,Sunscreen
Cyclononasiloxane, Octadecamethyl-	Antimicrobial,Cancer Presentive,Chemo Presentive
2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16,18,18,2 Dodecamethylcyclohexasiloxane	**Anti-Proliferative**
Hexasiloxane, Tetradecamethyl-	Nematiside,Antiantrogenic,Anticoranary, Antieczemic

****Source: Dr. Duke's Phytochemical and Ethno botanical Databases.**

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns.The spectrum of the unknown component was compared with the spectrum of theknown components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained. Among the identified phytochemicals, n-hexadecanoic acid have the property of antioxidant antimicrobial, larvicidal activities and liver disorder (Falodun, *et al*., 2009; Gopalakrishnan and Kalaiarasi, 2013). The phenolic constituents of the extracts of *Mentha spicata* namely phytol was reported for its antimicrobial and antiviral activities, strong antioxidant and antitumor action (Mckay and Blumberg, 2006).

Ashok kumar and Vijayalakshmi (2001) investigated the GC-MS chromatogram of the ethanolic extract of *P.granatum* peel showed 26 peaks indicating the presence of twenty six phytochemical constituents. On comparison of the mass spectra of the constituents with the NIST08, WILEY8 and FAME libraries the twenty six phytoconstituents were characterized and identified. The major phytochemical constituent's mass spectra are Glycerin, Hydroxymethylfurfurole, Guanosine and Pyrogallol.

The GC-MS analysis of *Caesalpinia italica* leaves revealed the presence of seventeen compounds. The identified compounds possess many biological properties. For instance, 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- Linolenic acid possesses anti-inflammatory, insectifuge, hypocholesterolemic, cancer preventive, nematicide, hepatoprotective, antihistaminic, antieczemic, antiacne, 5-alpha reductase inhibitor, antiandrogenic, antiarthritic and anticoronary properties. n-Hexadecanoic acid - palmitic acid can be an antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant activities and hemolytic 5-alpha is a reductive inhibitors. Phytol- Diterpene is an antimicrobial, anticancer, anti-inflammatory and diuretic agent (Praveen kumar *et al.*, 2012). 9, 12, 15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-, n-Hexadecanoic acid, 1,2-Benzenedicarboxylic acid and di-isooctyl ester were present in *Caesalpinia sappan* ethanol extract (Sarumathy *et al.*, 2011). Similar types of compounds were identified among the twenty five compounds of this present study.

Santhosh Kumar*et al*., (2014) Gas chromatography and mass spectrometry analysis of whole plant extract carried out with instrument GC-MS-QP. The methanol extracts of *Adiantum capillusveneris* to identified thirty seven bioactive compounds which are major compound such as 5-7A-Isoprpenyl-4, 5-Dimethyloctahydro-1h-inden-4yl)-3-methyl-2-penta, (24.49%), n-hexadecanoic acid (18.29%) and gamma-sitosterol (10.61%), cis-vaccenic acid(9.25%), 5-7A-Isopropenyl-4,5-Dimethyl-octahydro-inden-4-yl)-3-methyl-pent-2-EL(2.63%), Tetrdecanoic acid (2.20%) and Phenanthrene, 9-dodecyltetradecahydro (2.15%). The lowest percentage of peak area 0.25% and their compound as 2-methoxy-4-propyl, among these bioactive compounds are including Vitamin E (1.58%) also present**.**This identified compound are

having in antioxidant, antimicrobial, anti-inflammatory, diuretic and analgesic properties also these thirty seven bioactive compounds are biologically significance in many activities.

ANTI CANCER ACTIVITY

Cytotoxicity screening models provide important preliminary data to help selecting plant extracts with potential antineoplastic properties for future work (Cardellina, 1999). It is of interest that the extract of the plants showed cytotoxicity against cancer cell line, and, if this also occurs *in vivo*, the use of these plants by traditional healer for the treatment of cancer patients would have some scientific support. Several plant species rich in flavonoids are reported having disease preventive and therapeutic properties. This observation is of particular importance since flavonoids are ingredients of many vegetables and fruits and the association of vegetable and fruit consumption with reduced cancer risk has been reported (Ramos, 2007).

The results for cell growth inhibition by the extract against MCF 7 lines for various concentrations are shown in table 4 and fig 2 and3. As the concentration increases there is an increase in the cell growth inhibition but is found to be highest inhibition with only 91.11 % growth inhibition at 500 µg (Plate 1). The IC_{50} value was 155.40 µg/ml. Cytotoxic activity recorded in the present study is in accordance with this finding, since the phytochemical evaluation indicated the presence of flavonoids in plant species with promising activity.

he results obtained showed that hydroalcoholic extract of T. *divaricata* had a very moderate anticancer activity which was supported by a number of studies as follows: *Clerodendrum phlomidis* crude extracts of petroleum ether, ethyl acetate, chloroform and ethanol obtained from the root of the plant were tested for cytotoxic activity on Mouse embryonic fibroblasts cell line (NIH 3T3) and HeLa cell lines using MTT assay where ethanol extract had no cytotoxic activity and the other extracts had moderate to weak cytotoxic activity on both the cell lines (Sathish *et al*., 2011). In another study four trifoliate plant extracts in different solvents were tested for cytotoxic activity against HeLa cell lines and MCF7 cell lines and extract showed less significant activity against HeLa cell lines but showed good activity against MCF7 (Pranay Dogra, 2009). In a research the methanolic extracts of *Artocarpus heterophyllus* was tested for anticancer activity by MTT assay on different cell lines like HEK293, A549, HeLa and MCF-7 .

Table 4 Percentage cell growth inhibition of *P.granatum* peel extract on MCF 7cell line by MTT assay

S.No.	Concentrations (µg/ml)	Absorbance (Optical density)	Cell Viability (%)	Cell growth inhibition (%)
1.	100	0.674	76.76	23.23
2.	200	0.225	25.62	74.37
3.	300	0.160	18.22	81.77
4.	400	0.116	13.21	86.78
5.	500	0.070	8.88	91.11
	Cell Control	0.878	100	0
Half Inhibition Concentration (IC_{50})				155.40µg/ml

Fig.2: Percentage of cell growth inhibition of *P.granatum* peel extract on MCF 7cell line by MTT assay

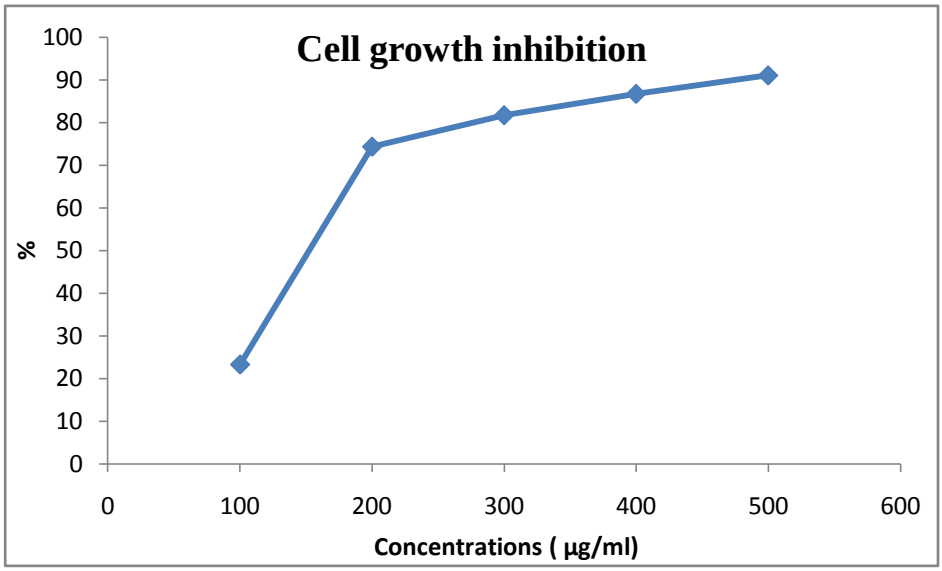


Fig. 3 Percentage of cell viability of *P.granatum* peel extract on MCF 7cell line by MTT assay

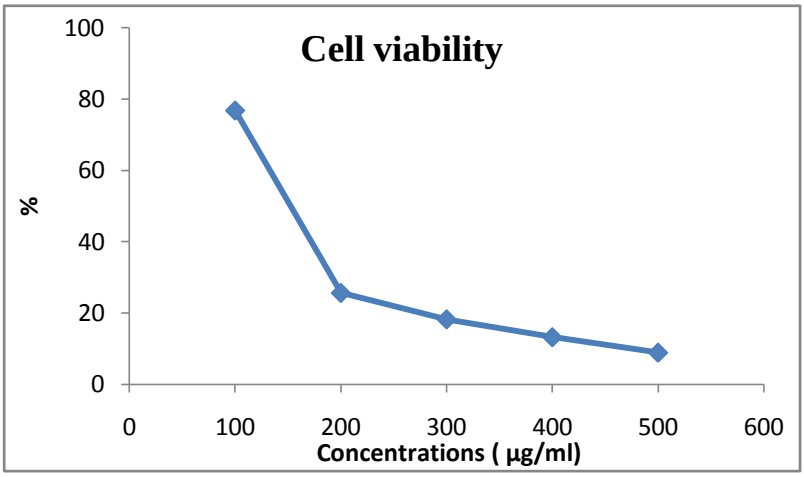
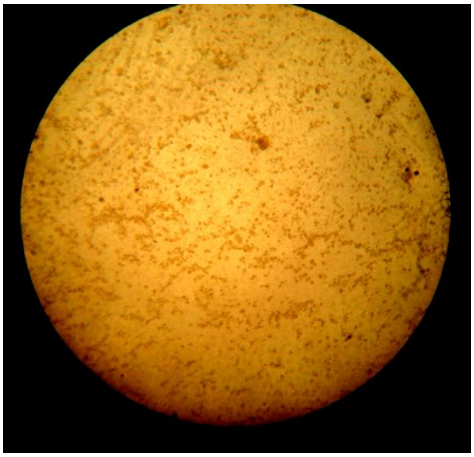


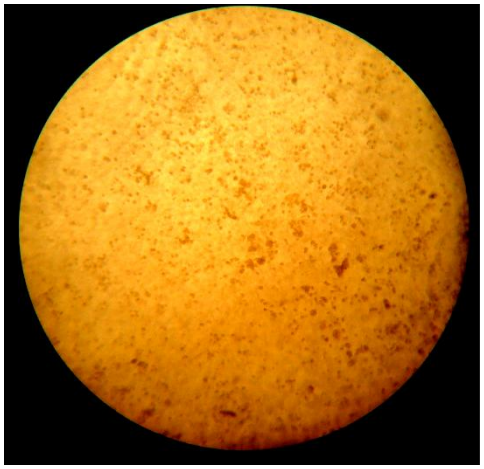
Plate. 1: Cell growth inhibition of *P.granatum* peel extract on MCF 7cell line by MTT assay

Group I (100%)

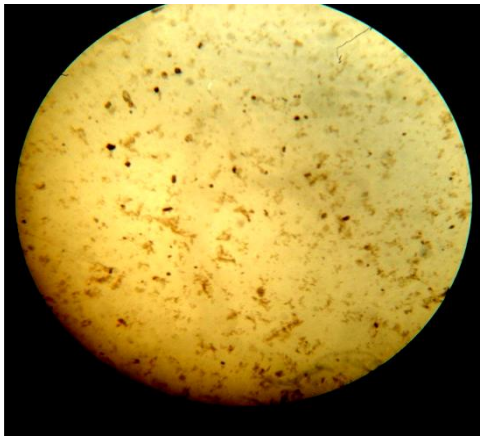
Group II (200%)



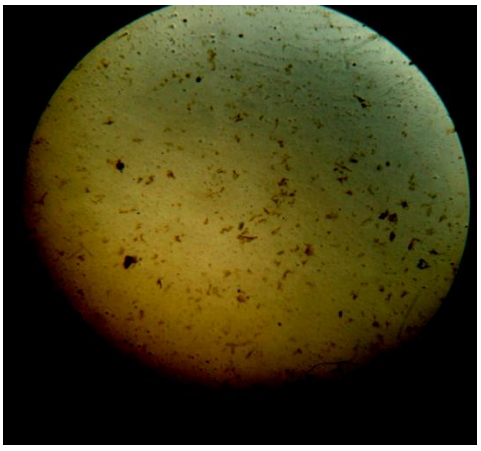
Group III (300%)



Group IV (400%)



Group V (500%)



The present results showed that *P. granatum* peel extract might be a potential alternative agent for human breast cancer therapy. Hence, it is anticipated That *P. granatum* peel would be a useful pharmaceutical material to treat breast cancer. Futureresearch should focus on the molecularmechanism of *P. granatum* peel anticancer action. Over all the *P. granatum* peel extract as a source of phytochemicals and possessed potential anticancer activity in cancer cell line. There is a need for further investigation of this plant in order to identify and isolate its active anticancer principle(s).

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